DNA DEMETHYLASE, THERAPEUTIC AND DIAGNOSTIC USES THEREOF

BACKGROUND OF THE INVENTION

5   (a) Field of the Invention

The invention relates to a novel enzyme, DNA demethylase, therapeutic and diagnostic uses thereof.

(b) Description of Prior Art

Many lines of evidence have established that
10   modification of cytosine moieties residing in the dinu-
cleotide sequence CpG in vertebrate genomes is involved
in regulating a number of genome functions such as
parental imprinting, X-inactivation, suppression of
methylation of ectopic genes and differential gene
15   expression (Szyf, M. (1996) Pharmacol. Ther. 70, 1-37).
DNA methylation performs its function of differentially
marking genes because the distribution of methylated
CpGs is tissue- and site- specific forming a pattern of
methylation (Szyf, M. (1996) Pharmacol. Ther. 70, 1-
20   37).   It is clear that the pattern of methylation is
fashioned by a sequence of methylation and demethyla-
tion events (Brandeis, M. et al. (1993) Bioassays 15,
709-713) during development and is maintained in the
fully differentiated cell (Razin, A. et al. (1980) Sci-
25   ence 210, 604-610).   While it was originally suggested
that DNA demethylation is accomplished by a passive
loss of methyl groups during replication (Razin, A. et
al. (1980) Science 210, 604-610), it is now clear that
an active process of demethylation occurs in embryonal
30   cells (Frank, D. et al. (1991) Nature 351, 239-241), in
differentiating cell lines (Razin, A. et al. (1986)
Proc. Natl. Acad. Sci. USA 83, 2827-2831; Szyf, M. et
al. (1985) Proc. Natl. Acad. Sci. USA 82, 8090-8094)
and in response to estrogen treatment (Saluz, H.P. et
35   al. (1986) Proc. Natl. Acad. Sci. USA 83, 7167-7171).
Two modes of demethylation have been documented: site

specific demethylation that coincides in many instances with onset of gene expression of specific genes and a general genome wide demethylation that occurs during early development *in vivo* during cellular differentia-
5 tion and in cancer cells (Feinberg, A.P. et al. (1983) Nature *301*, 89-92; Razin, A. et al. (1986) Proc. Natl. Acad. Sci. USA *83*, 2827-2831). The global demethyla-tion is consistent with the hypothesis that a general demethylase activity which is activated at specific
10 points in development or oncogenesis exists. It has been hypothesized that one mechanism regulating the pattern of methylation is the control of expression of methyltransferase (Szyf, M. (1991) Biochem. Cell Biol. *69*, 764-767) and demethylase activities (Szyf, M.(1994)
15 Trends Pharmacol. Sci. 7, 233-238). Although exten-sive information has been obtained on the enzymatic activity responsible for methylation and the regulation of its expression in the last two decades (Szyf, M. (1996) Pharmacol. Ther. *70*, 1-37), the identity of the
20 demethylase has remained a mystery. It is clear how-ever that to fully understand how patterns of methyla-tion are formed and maintained and to determine their role in development, physiology and oncogenesis, one has to identify the demethylase enzyme(s). Two main
25 difficulties have inhibited the identification of this enzyme. First, it is believed that demethylation of a methylated cytosine is chemically highly unlikely since it involves breaking a very stable C-C bond. Second, demethylation occurs at very defined stages in develop-
30 ment (Brandeis, M. et al. (1993) Bioassays *15*, 709-713) and identifying an adequate tissue source for this enzyme is critical.

Whereas no *bona fide* demethylase has been iden-tified to date, alternative biochemical mechanisms
35 involving exchange of methylated cytosines with non-

methylated cytosines have been described. One previously proposed mechanism is removal of the methylated base by a glycosylase and its replacement with a non-methylated nucleotide utilizing an "excision-repair" mechanism (Razin, A. et al. (1986) Proc. Natl. Acad. Sci. USA *83*, 2827-2831). Glycosylase activities that can remove methylated cytosines from DNA have been demonstrated by Vairapandi and Duker (Vairapandi, M. et al. (1993) *Nucl. Acids Res. 21*, 5323-5327) and more recently by Jost (Jost, J. P. et al. (1995) J. Biol. Chem. *270*, 9734-9739). However it is not clear whether this activity is responsible for the general demethylation observed in cellular differentiation. The fact that the activity identified by Jost acts specifically on hemimethylated sequences (which is not the natural substrate in most cases) and can remove thymidines as well as 5-methylcytosines, supports a repair function for this glycosylase-demethylase (Jost, J. P. et al. (1995) J. Biol. Chem. *270*, 9734-9739). An alternative mechanism involving a RNA dependent activity has been recently described by Weiss et al. (Weiss et al., 1996). This proteinase-insensitive RNA dependent activity has been shown to catalyze the excision and replacement of a methylated CpG dinucleotide with a nonmethylated CpG dinucleotide that is contained in a DNA-RNA hybrid molecule (Weiss, A. et al. (1996) Cell *87*, 709-718). This activity which was identified in differentiating cells in culture was proposed to be involved in demethylation during development. These previous findings demonstrate that the common accepted model in the filed has been that a *bona fide* demethylase does not exist.

It has been previously proposed that the extensive hypomethylation observed in cancer cells might be a consequence of activation of demethylase activity by

oncogenic pathways (Szyf, M.(1994) Trends Pharmacol.
Sci. 7, 233-238; Szyf, M. et al. (1995) J. Biol. Chem.
270, 12690-12696).   In accordance with this hypothesis
we have shown that ectopic expression of v-Ha-ras had
5   induced demethylation activity in the cells (Szyf, M.
et al. (1995) J. Biol. Chem. *270*, 12690-12696). Using
an assay that directly measures the conversion of $3'^{32}P$
labeled methyl dCMP (mdCMP) into dCMP, we have shown
that nuclear extracts prepared from P19-Ras transfec-
10   tants bear high levels of demethylase activity (Szyf,
M. et al. (1995) J. Biol. Chem. *270*, 12690-12696).
Building on this observation, we hypothesized that can-
cer cell lines were a good source for demethylase.
However, it is not evident that Ras expression in p19
15   cells does reflect the situation in cancer cells. P19
is an embryonic cell and expression of Ras might be
differentiating them.

     It would be highly desirable to be provided with
a *bona fide* DNA demethylase (DNA dMTase) to alter
20   developmental programs for therapeutic and biological
use.

SUMMARY OF THE INVENTION

     In accordance with the present invention, we
25   demonstrate the purification of a *bona fide* DNA demeth-
ylase (DNA dMTase) from a human lung cancer cell line
A549, determine its kinetic parameters and substrate
specificity. The DNA dMTase activity identified in
this study converts methyl-dCMP (mdCMP) residing in the
30   dinucleotide sequence mdCpG into dCMP whereas the
methyl group is released as a volatile residue which
was identified to be methanol. The activity is puri-
fied away from any trace amounts of dCTP, is insensi-
tive to the DNA polymerase inhibitor ddCTP, is not
35   affected by the presence of methyl dCTP (mdCTP) in the

reaction and does not exhibit exonuclease or glyco-sylase activities.   The identification of this new enzyme points out to new directions in our understand-ing of how DNA methylation patterns are formed and
5   altered.

One aim of the present invention is to provide a *bona fide* DNA demethylase (DNA dMTase).

In accordance with the present invention there is provided a DNA demethylase enzyme having about
10 40 KDa, and wherein the DNA demethylase enzyme is over-expressed in cancer cells and not in normal cells.

In accordance with the present invention there is provided a cDNA encoding human demethylase which comprises a sequence set forth in SEQ ID NO:1.
15 In accordance with the present invention there is provided two mouse cDNAs homologous to the human cDNA, wherein the cDNA encoding mouse demethylase hav-ing a sequence set forth in SEQ ID NOS:5-7.

In accordance with the present invention there
20 is provided a different human cDNA which encodes a pro-tein homologous to the human demethylase having a sequence set forth in SEQ ID NO:3.

In accordance with the present invention there is provided the use of the expression of demethylase
25 cDNAs to alter DNA methylation patterns of DNA *in vitro* in cells or *in vivo* in humans, animals and in plants.

The demethylase cDNAs expression may be under the direction of mammalian promoters, such as CMV.

The demethylase cDNAs expression may be under
30 plant specific promoters to alter methylation in plants and to allow for altering states of development of plants and expression of foreign genes in plants.

The demethylase cDNAs expression may be in the antisense orientation to inhibit demethylase in cancer
35 cells for therapeutic processes.

The expression of demethylase cDNA in mammalian cells may be to alter their differentiation state and to generate stem cells for therapeutics, cells for animal cloning and to improve expression of foreign genes.

In accordance with the present invention there is provided the use of the expression of demethylase cDNAs in bacterial or insect cells for production of large amounts of demethylase.

In accordance with the present invention there is provided the use of the expression of demethylase cDNAs for the production of protein in vertebrate, insect or bacterial or plant cells, such as antibodies against demethylase.

In accordance with the present invention there is provided the use of the sequence of demethylase cDNAs as a template to design antisense oligonucleotides and ribozymes.

In accordance with the present invention there is provided the use of the predicted peptide sequence of demethylase cDNAs to produce polyclonal or monoclonal antibodies against demethylase.

In accordance with the present invention there is provided the use of expression of cDNAs in two hybrid systems in yeast to identify proteins interacting with demethylase for diagnostic and therapeutic purposes.

In accordance with the present invention there is provided the use of expression of cDNAs in bacterial, vertebrate or insect cells to produce large amounts of demethylase for obtaining a x-ray crystal structure and for high throughput screening of demethylase inhibitors for therapeutics and biotechnology.

In accordance with the present invention there is provided a volatile assay for high throughput

screening of demethylase inhibitors as therapeutics and
anticancer agents which comprises the steps of:

   a) using transcribed and translated demethylase
      cDNAs *in vitro* to convert methyl-cytosine pres-
5   ent in methylated DNA samples to cytosine pres-
      ent in DNA and volatilize methyl group;

   b) determining the absence or minute amount of
      volatilize methyl group as an indication of an
      active demethylase inhibitor.

10   In accordance with the present invention there
is provided a volatile assay for the diagnostics of
cancer in a patient sample which comprises the steps
of:

   a) determining demethylase activity in patient sam-
15   ples by assaying conversion of methyl-cytosine
      present in methylated DNA to cytosine present in
      DNA and its volatilization as methyl groups
      released as methanol;

   b) determining the presence or minute amount of
20   volatilized methyl released as methanol groups
      as an indication of cancer in the patient sam-
      ple.

       In accordance with the present invention there
is provided the use of an antagonist or inhibitor of
25 DNA demethylase for the manufacture of a medicament for
cancer treatment, for restoring an aberrant methylation
pattern in a patient DNA, or for changing a methylation
pattern in a patient DNA.

       Such an antagonist is a double stranded oligonu-
30 cleotide that inhibits demethylase at a Ki of 50nM,
such as $\begin{bmatrix} C^mGC^mGC^mGC^mG \\ G^mCG^mCG^mCG^mC \end{bmatrix}_n$

       The inhibitors include, without limitation an
anti-DNA demethylase antibody, an antisense of DNA
35 demethylase or a small molecule such as any derivative
of imidazole.

The change of the methylation pattern may activate a silent gene. Such an activation of a silent gene permits the correction of genetic defect such as found for β-thalassemia or sickle cell anemia.

The DNA demethylase of the present invention may be used to remove methyl groups on DNA *in vitro* such as needed for cloning DNA.

The DNA demethylase of the present invention or its cDNAs may be used, for changing the state of differentiation of a cell to allow gene therapy, stem cell selection or cell cloning.

The DNA demethylase of the present invention or its cDNAs may be used, for inhibiting methylation in cancer cells using vector mediated gene therapy.

In accordance with the present invention there is provided an assay for the diagnostic of cancer in a patient, which comprises determining the level of expression of DNA demethylase by either RT-PCT, ELISA or volatilization assay of the present invention in a sample from the patient, wherein overexpression of the DNA demethylase is indicative of cancer cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A to 1B illustrate the purification of demethylase (DNA dMTase) from human A549 cells;

Figs. 2A and 2C illustrate that DNA dMTase is a protein inhibited by RNA and not by ddCTP, mdCTP;

Figs. 2B and 2D illustrate the kinetics of DNA dMTase activity;

Figs. 3A to 3C illustrate the product of DNA dMTase activity is cytosine and it exhibits no exonuclease or glycosylase activity;

Figs. 4A-4C illustrate the demethylation reaction releases methanol as a volatile residue;

Fig. 4D illustrates the transfer of a proton from water to regenerate cytosine;

Figs. 4E-4F illustrate that the volatile product is methanol;

5   Fig. 5 illustrates the suggested demethylation reaction;

Figs. 6A-6D illustrate the substrate Specificity of DNA dMTase;

Figs. 7A-7D illustrate chromatographic isolation

10   of dMTase from human A549 cells;

Figs. 8A-8B illustrate the alignment between the MDB domain of MeCP2 and demethylase and the predicted amino acid sequence of human demethylase;

Fig. 8C illustrates the mRNA encoded by demethy-

15   lase;

Figs. 9A-9F illustrate the cDNA and their predicted amino acid of demethylases and homologues of the present invention (SEQ ID NOS:1-8);

Figs. 10A-B illustrate a mammalian expression

20   vector of dMTase and in vitro translated dMTase polypeptide;

Fig. 10C illustrates that in vitro translated DNA dMTase releases volatile methyl residues from methylated DNA;

25   Fig. 10D illustrates that in vitro translated DNA dMTase transform methylated cytosines to cytosines;

Fig. 11A illustrates that transiently transfected demethylase releases volatile residues from methylated DNA;

30   Fig. 11B illustrates the polypeptide expressed from transiently transfected demethylase;

Figs. 11C-11E illustrate that transiently transfected demethylase transforms methylated cytosines to cytosines in a protein dependent manner;

Fig. 11F illustrates that the transformation of methylated cytosine to cytosine by transiently trans- fected demethylase depends on the concentration of sub- strate;

Fig. 12A illustrates that transiently trans- fected demethylase catalyzes the transfer of a proton from tritiated water to regenerate cytosine;

Fig. 12B illustrates that the cloned demethylase releases methanol from methylated DNA;

Figs. 13A-13C illustrate that the cancer cells express demethylase activity whereas normal cells do not;

Fig. 13D illustrates that demethylase mRNA is highly express in cancer cells;

Fig. 14A illustrates demethylase bacterial ret- roviral and mammalian expression vector;

Fig. 14B illustrates inhibition of demethylase activity by a specific inhibitor;   *growth*

Fig. 14C illustrates inhibition of tumorigenesis *in vitro* by an inhibition of demethylase;

Fig. 15 illustrates inhibition of tumorigenesis in cell culture by induced expression of demethylase antisense vector;

Fig. 16 illustrates the inhibition of demethy- lase by a small molecule inhibitor imidazole; and

Fig. 17 illustrates a model for the inhibition of cancer growth by an inhibition of demethylase.

DETAILED DESCRIPTION OF THE INVENTION

The pattern of methylation is fashioned during development by a sequence of methylation and demethyla- tion events. The identity of the demethylase has remained a mystery and alternative biochemical activi- ties have been shown to demethylate DNA but no activity that can truly remove methyl groups from DNA has been

shown to date. Utilizing human lung carcinoma cells as a source for demethylase activity we demonstrate that mammalian cells bear a *bona fide* DNA demethylase (DNA dMTase) activity. DNA dMTase transforms methyl-C to C by catalyzing replacement of the methyl group on the 5 position of C with a hydrogen derived from water. DNA dMTase demethylates both fully methylated and hemimethylated DNA, shows dinucleotide specificity and can demethylate mdCpdG sites in different sequence contexts. This enzyme is different from previously described demethylation activities: it is proteinase sensitive, activated by RNase and releases different products.

DNA dMTase is a novel enzyme showing a new and unexpected activity that has not been previously described in any organism. The finding of a *bona fide* demethylase, points out new directions in our understanding of the biological role of DNA methylation.

In spite of the fact that it was previously shown that Ras expression in p19 cells can induce demethylation activity. It was not clear whether this demethylation activity is indeed a *bona fide* demethylase. One would predict that demethylase is present in embryonal cells. It was surprising to see that demethylation activity is present in cancer cells. The finding of high levels of demethylase in A549 cells is indeed an unexpected discovery.

In accordance with the present invention, it is shown and demonstrated that demethylation occurs by removal of a methyl group from methylated cytosine in DNA, that a hydrogen from water replaces the methyl group at the 5' position, that the resulting methyl group reacts with the remaining hydroxyl from water to generate methanol which volatilizes (Fig. 4E-F). Thus,

bona fide demethylation of DNA involves the following reaction:

$$CH_3\text{-cytosine-(DNA)}+H\text{-OH} \xrightarrow{\text{demethylase}} H\text{-cytosine} + CH_3\text{-OH}$$
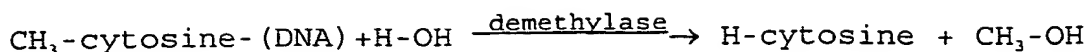
5   The cDNA cloned in accordance with the present invention is the demethylase since it can convert methyl-cytosines in DNA to cytosines and volatilize the methyl groups on DNA when transcribed and translated *in vitro* which are released as methanol. This is a novel

10   cDNA encoding a biochemical activity that has been not described before.

In accordance with the present invention, there is shown a model for the inhibition of cancer growth by an inhibition of demethylase (Fig. 17).

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EXPERIMENTAL PROCEDURES

Cell Culture

A549 Lung Carcinoma cells (ATCC: CCL 185) were grown in Dulbecco's modified Eagle's medium (with low

20   glucose) supplemented with 10% fetal calf serum, 2 mM glutamine, 10 U/ml cifrofloxacin. Human Skin Fibro-blasts #72-213A MRHF were obtained from BioWhittaker, Bethesda and were grown in Dulbecco's modified Eagle's medium supplement with 2% fetal calf serum, 2 mM gluta-

25   mine. H446 Lung carcinoma cells (ATCC: HTB 171) was grown in RPMI 1640 medium with 5% fetal calf serum.

Preparation of nuclear extract

Nuclear extracts were prepared from A549 cultures at near confluence as previously described (Szyf

30   et al., 1991; Szyf et al.,1995). The cells were tryp-sinized, collected and washed with phosphate-buffered saline and suspended in buffer A (10 mM Tris, pH 8.0, 1.5 mM MgCl$_2$, 5mM KCl, 0.5% NP-40) at the concentration of 10^8 cells per ml for 10 min. at 4°C. Nuclei were

35   collected by centrifugation of the suspension at 1000 g

for 10 minutes. The nuclear pellet was resuspended in buffer A (400 μl) and collected as described in the experimental procedures. A nuclear extract was prepared from the pelleted nuclei by suspending them in buffer B (20 mM Tris, pH 8.0, 25% glycerol, 0.2 mM EDTA and 0.4 mM NaCl) at the concentration of 3.3×10^8 nuclei per ml and incubating the suspension for 15 min. at 4°C. The nuclear extract was separated from the nuclear pellet by centrifugation at 10,000g for 30 minutes. Nuclear extract were stored in -80°C for at least two months without loss of activity.

Chromatography on DEAE-Sephadex

A freshly prepared nuclear extract (1 ml , 1.1 mg) was passed through a Microcon™ 100 spin column, the retainant was diluted to a conductivity equivalent to 0.2 M NaCl in buffer L and applied onto a DEAE-Sephadex column (Pharmacia) (1.0 x 5 cm) that was preequilibrated with buffer L (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$) containing 0.2 M NaCl at a flow rate of 1 ml/min. The column was then washed with 15 ml of the starting buffer (buffer L + 0.2 M NaCl) and proteins were eluted with 5 ml of a linear gradient of NaCl (0.2-5.0 M). 0.8 ml fractions were collected and assayed for demethylase activity after desalting through a Microcon™ 10 spin column (Amicon) and resuspension of the retainant in 0.8 ml buffer L. DNA demethylase eluted between 2-5.0 M NaCl.

Chromatography on S-Sepharose

Active DEAE-Sepharose column fractions were pooled, adjusted to 0.1 M NaCl by dilution and loaded onto an S-sepharose column (Pharmacia) (1.0 x5 cm) which had been preequilibrated with buffer L containing 0.2 M NaCl at a flow rate of 1 ml/min. Following washing of the column as described in experimental procedures, the proteins were eluted with 5 ml of a linear

NaCl gradient (0.2-5.0M). 0.5 ml fractions were col-
lected and assayed for DNA demethylase activity after
desalting and concentrating to 0.2 ml using a Microcon™
10 spin column. DNA demethylase activity eluted around
5   5.0 M NaCl.

Chromatography on Q-Sepharose

Active fractions from S-sepharose column were
pooled, adjusted to 0.2 M NaCl by dilution and applied
onto a Q-sepharose (Pharmacia) column (1.0 x5 cm) which
10 had been equilibrated as described in the experimental
procedures at a flow rate of 1 ml/min. The column was
washed and the proteins were eluted with a linear NaCl
gradient (0.2- 5.0 M). Fractions (0.5 ml) were col-
lected, assayed for demethylase activity after desalt-
15 ing and concentrating to a final volume of 0.2 ml as
described in the experimental procedures. The demethy-
lase activity eluted around 4.8-5.0 M NaCl.

Gel-Exclusion Chromatography on DEAE-Sephacel

The pooled fractions of Q-sepharose column were
20 adjusted to 0.2 M NaCl, loaded onto a 2.0 x 2.0 cm
DEAE-Sephacel column (Pharmacia) and eluted with 10 ml
of buffer L containing 0.2 M NaCl. The fractions (0.8
ml) were collected and assayed after concentration to
about 180 μl with a Microcon™ 10 spin column for DNA
25 demethylase activity. The activity was detected at
fraction 4, which is very near the void volume
(~200kDa).

Assay of DNA demethylase activity

To directly assay DNA demethylase activity in
30 vitro two independent methods were applied.

(A) To assay the conversion of methyl-dCMP (mdCMP) to
dCMP we used a previously described method (Szyf et
al., 1995). Briefly, α^{32}P labeled, fully methylated
poly[mdC^{32}PdG]n substrate was prepared as follows. One
35 hundred ng of a double-stranded fully methylated

(mdCpdG) oligomer (Pharmacia) were denatured by boiling, which was followed by partial annealing at room temperature. The complementary strand was extended with Klenow fragment (Boehringer Mannheim) using

5   methyl-5-dCTP (mdCTP, 0.1 mM) (Boehringer Mannheim) and $[\alpha-^{32}P]$ GTP (100 μCi, 3000 Ci/mmol), and the unincorporated nucleotides were removed by chromatography through a NAP-5 column (Pharmacia). The NAP-5 chromatography was repeated to exclude minor contamination

10 with unincorporated nucleotides. As a control a non-methylated poly$[dC^{32}pdG]$n substrate was similarly prepared except that a nonmethylated dCpdG oligomer served as a template and dCTP was used in the extension reaction. The column fractions (30 μl), described in the

15 experimental procedures were incubated with 1 ng of poly$[mdC^{32}pdG]$n substrate for 1 hour at 37°C in a buffer L containing 25% glycerol (v/v) and 5 mM EDTA. The reacted DNA as well as a nonmethylated poly$[dC^{32}pdG]$n and methylated $[mdC^{32}pdG]$n nonreacted con-

20 trols were purified by phenol/chloroform extraction and subjected to micrococcal nuclease digestion (100 μg at 10 μl) and calf spleen phosphodiesterase (2μg) (Boehringer) (Pharmacia) to 3' mononucleotides for 15 hours at 37°C. The digestion products were loaded onto

25 a thin layer chromatography plate (TLC) (Kodak, 13255 Cellulose), separated in a medium containing, 132 ml Isobutyric acid:40 ml water: 4 ml ammonia solution, autoradiographed and the intensity of the different spots was determined using a phosphorimager (Fuji, BAS

30 2000). ^{32}P labeled substrates and tritium labeled substrates were phosphoimaged using BAS 2000 plate and BAS-TR2040 phosphorimager plate respectively.

(B) The second method determined removal of methylated residues from methylated DNA by measuring disappearance

35 of $^{3}H-CH_{3}$ or $^{14}C-CH_{3}$ from the reaction mixture. 100 ng

of poly [dCdG]n double stranded DNA was methylated
using SssI methylase (New England Biolabs) and an
excess of [^{3}H-methyl AdoMet (80 Ci/mmol; New England
Nuclear)]. The tritiated methyl group containing DNA

5   was purified from labeled AdoMet using NAP-5 column
chromatography. All column purified fractions of DNA
demethylase were assayed using the tritiated substrate.
In a typical assay, 1 ng of DNA was incubated (at a
specific activity of 4 x10^{6}dpm/mg)with 30 μl of column

10   fraction for one hour at 37°C in buffer L. To deter-
mine the number of methyl groups remaining in the DNA
following incubation with the different fractions, 250
μl of water were added and the mixture was incubated at
65°C for 5 minutes. One hundred μl of the reaction

15   mixture were withdrawn for liquid scintillation count-
ing. Controls received similar treatment except that
in place of a column fraction, an equal volume of
buffer L was added. The number of methyl groups that
were removed from the DNA by the different fractions

20   was determined by subtracting the remaining counts in
each of the fractions from the counts remaining in the
control. All tests were carried out in triplicates.
The results are presented as picomole methyl group
removed. One unit of DNA dMTase activity is defined

25   as: amount of enzyme that releases one picomole of
methyl group from methylated dCpdG substrate in one
hour at 37°C.

Methyl removal assay using double-labeled substrates

To determine whether the methyl group leaves the

30   DNA and not any non-specific removal of tritium, we
prepared SK plasmid DNA containing a tritiated hydrogen
at the 6' position of cytosine and thymidine by growing
the plasmid harboring bacteria in the presence of deoxy
[6-^{3}H] Uridine (22 Ci/mmol; Amersham) (10μCi/ml). The

35   [6-^{3}H]-cytosine containing pBluescript SK(+) was puri-

fied according to standard protocols and was methylated using an excess of [^{14}C-methyl] AdoMet (59 mCi/mmol; Amersham) (10 μCi per 100 μl reaction)and SssI methylase. The double labeled DNA substrate was purified

5 twice on a NAP-5 column. 15 μl of DNA dMTase were incubated with 1 ng of double labeled DNA (specific activity of 2000 dpm/ng) for 1 hour at 37°C. Following incubation, the remaining ^{14}C versus ^{3}H counts were determined as described in the experimental procedures

10 by scintillation counting (Wallac). The ^{14}C counts were normalized against ^{3}H counts. The controls received similar treatment except that instead of DNA dMTase, an equal amount of distilled water was added to them.

To determine the number of ^{3}H-CH$_3$ in the gaseous

15 phase, 1 ng of ^{3}H-CH$_3$ poly [dCpdG] DNA were incubated with DNA dMTase overnight in a sealed tube (Pierce, Illinois, USA). 0.8 ml of air were removed from the tube using a gas tight syringe (Hamilton, Reno, Nevada) and injected into a sealed gas tight scintillation vial

20 containing 10 ml OptiPhase scintillation fluid (Wallac, UK) and counted. As a control the DNA was incubated with an equal volume of buffer L and treated similarly.

Synthesis of other methylated dC dinucleotides

Poly [mdC^{32}pdA] and [mdC^{32}pdT] substrates were

25 prepared as follows. About 0.5 μg of 20 mer oligonucleotides 5'(GG)103', 5'(GT)103' and 5'(GA)103' were boiled and annealed at room temperature with oligonucleotide 5'CCCCCC3', 5'CACACA3' and 5'CTCTCT3' respectively. The complementary strand was extended with

30 Klenow fragment using m5dCTP (Boehringer Mannheim) and either [α^{32}P] dATP (100μCi, 3000Ci/mmol) or [α^{32}P] dTTP (100 μCi, 3000 Ci/mmol) respectively. The unincorporated nucleotides were removed by chromatography through a NAP-5 column. Hemimethylated mdCpG substrate

35 was prepared in a similar manner except that a nonmeth-

ylated poly dCpdG substrate (Boehringer) was used as template and m5dCTP and $[\alpha^{32}P]dGTP$ were used for extension as described in the experimental procedures.

Assay for nuclease and glycosylase activity

5    $[^{32}pmdCpdG]n$ substrate which included a labeled ^{32}P 5' to mdC was prepared as follows. About 100 ng of poly dCpdG DNA were boiled and partially annealed at room temperature. $[\alpha^{32}P]dCTP$ and cold dGTP were used for complementary strand extension as described in the

10   experimental procedures. The free nucleotides were separated using NAP-5 column chromatography. The purified $[^{32}pmdCpdG]n$ DNA was subjected to methylation by SssI methylase using 320 μM AdoMet. The DNA was repurified twice using a NAP-5 column. The methylated DNA (1

15   ng) was incubated with either 30 μl DNA dMTase, nuclear extract or buffer L. To determine whether $\alpha^{32}P$ labeled residue is excised from the DNA it was directly applied (3μl) onto a TLC plate. To determine whether the DNA was demethylated it was subjected to digestion with

20   snake venom phosphodiesterase (0.2 mg in a 10μl reaction volume) (Boehringer Mannheim) which attacks the 3'-OH group releasing 5'-mononucleotides. The resulting mononucleotides were separated on TLC plates and autoradiographed.

25   To test whether dCTP copurifies with DNA dMTase, which may be involved in activities other than *bona fide* demethylation, 20 μM of dCTP with 1 μl of $\alpha^{32}P$ labeled dCTP (3000 Ci/mmole) was loaded onto the column with nuclear extract. The ^{32}P counts were measured in

30   the flow through, washes and in the different fractions. About 1.1 million counts were loaded onto the DEAE-Sepharose column and were all recovered up to fraction 8.

To determine whether DNA dMTase contains a DNA

35   polymerase activity, DNA demethylase reactions were

performed in presence of 500 μM of ddCTP (Pharmacia) or 500 μM of m5dCTP (Boehringer Mannheim) at initial rate conditions.

To determine whether DNA dMTase is sensitive to
5   RNase or Proteinase K treatment, DNA dMTase was pre-treated for 1 h at 56°C with 200 μg/ml proteinase K (Sigma). A demethylation reaction was carried out with this pretreated fraction in the usual manner using both demethylation assays described in the experimental pro-
10   cedures. To test the effect of RNA digestion on the demethylation reaction, the fractions from different columns were treated with 100 μg/ml RNase A (Sigma).

Demethylation of pBluescript SK(+) Plasmid

About 4 μg plasmid pBluescript SK (Stratagene)
15   was subjected to methylation using SssI methylase. The methylated plasmid (4 ng) was incubated with 30 μl of DNA dMTase Fraction 4 of DEAE-Sephacel column under standard conditions, extracted with phenol: chloroform and precipitated with ethanol. About 1 ng of the plas-
20   mid were subjected to digestion with 10 units each of either of the restriction endonucleases EcoRII (GIBCO-BRL), DpnI, HhaI or HpaII (New England Biolabs) before and after methylation as well as after DNA dMTase treatment in a reaction volume of 10 μl for 2 hour at
25   37°C. Following restriction digestion the plasmids were extracted with phenol:chloroform, ethanol precipi-tated and resuspended in 10 μl. The plasmids were electrophoresed on a 0.8% (w/w) Agarose gel, trans-ferred onto a Hybond Nylon membrane and hybridized with
30   pBluescript SK(+) plasmid which was ^{32}P labeled by ran-dom-priming (Boehringer Mannheim).

Effect of Redox Reagents (NAD, NADH, NADP, NADPH and FeCl$_3$) on demethylase activity

The reagents were prepared at 100 μM concentra-
35   tion and added at a final concentration of 10 μM to a standard methyl removal assay under initial rate condi-

tions as described in the experimental procedures. The methyl removal activity in presence of each of the cofactors was compared to a control DNA dMTase reaction.

5   **Determination of kinetic parameters**

For determination of kinetic parameters, the demethylation reactions were performed using both assays (generation of dCMP and removal of methyl) as described in the experimental procedures except that
10 varying DNA concentrations from 0.1 nM to 2.5 nM were used in a total volume of 50μl including 30 μl of DNA dMTase. Since it has been established by previous experiments that the reaction proceeds for at least 3 hours, the initial velocity of reaction was measured
15 at one hour intervals. The velocity data was collected at each substrate DNA concentration range stated for both assays. The Km and Vmax values for DNA demethylase activity were determined from double reciprocal plots of velocity versus substrate concentration.

20 **Measurements of methanol production catalyzed by demethylase by gas chromatography**

Gas chromatography was performed with a Varian™ model 3400 GC equipped with a 30m Stabilwax™ column (0.053 cm i.d.: Restek Corporation). Nitrogen™ was
25 used as carrier gas at a flow rate of 32 ml/min, the injector and detector chambers were at 200 and 300°C respectively. The column was maintained at 40°C for 5 minutes after sample injection.

The demethylase reaction was performed in eppen-
30 dorf tubes kept within sealed scintillation vials with 300 μl of water as aqueous phase (in radioactive trapping experiments this was replaced by 300 μl of methanol). The demethylase reaction was initiated in buffer L (10 mM MgCl$_2$, 10 mM Tris-HCl pH 8.0) with 500 ng of
35 tritiated SK plasmid (6000 dpm/μl) and 100 μl of demethylase at 37°C. After overnight incubation at 37°C,

the aqueous phase surrounding the eppendorf tube was transferred to a fresh eppendorf tube, 2 µl of this mixture was injected in the gas chromatography using a gas tight syringe (Hamilton, Reno, Nevada).

Coupled *in vitro* transcription translation

The mRNAs encoded by the pcDNA 3.1/His Xpress demethylase constructs described above were transcribed and translated by coupled transcription-translation using Promega™ TNT reticulocyte lysate kit (according to manufacturer's protocol), 2 µg of each construct and 40µCi of [^{35}-S]methionine (1,000Ci/mmol, Amersham) in a 50µl reaction volume. To purify non labeled *in vitro* translated demethylase, coupled *in vitro* transcription and translation was performed as above but in the presence of cold methionine. The translation products were bound to a Probond™ nickel column (Invitrogen) and demethylase was eluted according to the manufacturer's protocol with increasing concentrations of imidazole. Demethylase is eluted at 350-500mM imidazole. The imidazole eluted demethylase was dialyzed and concentrated by lyophilization.

Gas chromatography coupled with Mass spectrometry (GC-MS) Analyses for identification of volatile product of demethylase catalyzed reaction as methanol

The demethylation reactions (volume 50 l) were run in conical vials having a total internal volume of 350 microlitres. The vials were closed with a teflon-lined screw cap and left at room temperature for 18 h. The vials were cooled in an ice bath, opened and 10 mg of NaCl and 50 microlitres of toluene were added. The vials were frequently shaken over a period of 1 h. The toluene phases were pipetted into clean vials in a manner to rigorously exclude water carry over. Anhydrous sodium sulfate (5 mg) was added to the toluene extracts to remove water, and the toluene phases were pipetted

into autoinjector vials for GC/MS analysis.   Aliquots
of 3 microlitres were analyzed under the following
instrumental conditions: Instrument: Hewlett-Packard
5988A;   Column: 30 m x 0.25 mm i.d. fused quartz capil-
5   lary with 0.25 micron DB-1 liquid phase, programmed
after an initial hold for 1 min at 70 deg at 5 deg/min
to 80 deg, then ramped ballistically to 280 deg for
bake-out for 5 min;   Injector and interface tempera-
tures: 250 deg;   Helium flow rate 1.5 ml/min; Mass
10 spectrometer: ion source 200 deg, 70 eV electron impact
ionization, scanning from m/z 10 to 50 in full scan
mode was begun 6 s after injection, and ceased at 1.5
min to avoid acquisition of the intense toluene solvent
peak.
15

**Human A549 cells bear a demethylase activity that could
be purified away from dCTP and DNA MeTase**

The use of an appropriate cellular source and a
direct assay for demethylase activity are obviously
20 critical.   As we have previously shown that demethylase
activity was induced in response to ectopic expression
of the Ras oncogene (Szyf et al., 1995) we reasoned
that cancer cells might bear high levels of demethy-
lase activity.   Based on preliminary studies demon-
25 strating the presence of high levels of demethylase
activity in the human lung carcinoma cell line A549, we
have chosen this cell line for our further studies and
purification steps.   Previous studies have used indi-
rect measures such as increased sensitivity to methyla-
30 tion-sensitive restriction enzymes as indicators of
demethylase activity (Weiss et al., 1996; Jost et al.,
1995).   To directly measure the conversion of 5-mdCMP
in DNA to dCMP, we have utilized a completely methyl-
ated ^{32}P labeled [mdC^{32}pdG]n double stranded oligomer
35 which we had previously described (Szyf et al., 1995).
Following incubation with the different fractions, the

DNA is purified and subjected to cleavage with micrococal nuclease to 3' mononucleotides. The 3' labeled mdCMP and dCMP are separated by thin layer chromatography (TLC) and the conversion of mdCMP to dCMP is

5 directly determined. This assay provides a stringent test for *bona fide* demethylation and discriminates it from previously described 5mCpC replacement activities (Jost *et al.*, 1995; Weiss *et al.*, 1996). The glycosylase-demethylase activity described by Jost et al.

10 (Jost *et al.*, 1995) will require the presence of a ligase activity and an energy source for replacement of mdC with C to be detected by our assay, whereas the demethylase activity described by Weiss et al. will not be detected since it replaces the intact $mdC^{32}pdG$ dinu-

15 cleotide with a cold dCpdG without altering its state of methylation (Weiss *et al.*, 1996).

Nuclear extracts were prepared from A549 cells, applied onto a DEAE-Sephadex column, eluted with a linear gradient from 0.2-5.0M NaCl and the fractions were

20 assayed for demethylase (dMTase) activity as described in the experimental procedures. As shown in Fig. 1(A) a clear peak of dMTase activity is eluted at the high salt fraction 10.

Conversion of methylated cytosine to cytosine:

25 Nuclear extracts prepared from A549 cells (1.1 mg) were passed through an AMICON™ 100 spin column. The retainant (98.56 mg, 0.2 mg/ml) was loaded onto a DEAE-Sepharose column, the different chromatographic column fractions eluted by a linear NaCl gradient (0.2-5M) were

30 desalted and (30 μl) incubated with 1 ng of $[mdC^{32}pdG]n$ double stranded oligomer for 1 hour at 37°C, digested to 3' mononucleotides and analyzed on TLC as described in the experimental procedures. Control methylated (ME) and nonmethylated (NM) $[dC^{32}pdG]n$ substrates were

35 digested to 3' mononucleotides and loaded on the TLC

plate to indicate the expected position of dCMP and mdCMP. The active fraction is indicated by an arrow. This fraction was loaded on S-Sepharose followed by Q-Sepharose and DEAE-Sephacel fractionation.

5   The first chromatography step purified the dMTase activity from the bulk of nuclear protein (Fig. 1B) and is a very effective purification step.

DNA dMTase activity as measured by the release of volatile methyl residues. The different column
10 fractions were incubated with 1ng (4 x 10^6 dpm/μg) of [^{3}H]-CH$_3$-[mdCpdG]n oligomer and the release of volatile methyl residues was determined (-) and presented as total dpn). The results are an average of three inde-pendent determinations. Protein concentration was
15 determined using the Bio-Rad Bradford kit (-). The elution profile of 20 μM of [^{32}P]-α-dCTP incubated with the protein was determined by scintillation counting of the different DEAE fractions (-) and presented as frac-tion of dCTP loaded on the column.

20   To exclude the possibility that the DNA dMTase activity detected in our assay is carried by the DNA MeTase, we assayed the fractions for DNA MeTase activ-ity using a hemimethylated DNA substrate as previously described (Szyf et al., 1991). As observed in Figure
25 1B DNA MeTase activity is detected in the second and third fractions, thus our fractionation separated DNA dMTase away from the DNA MeTase suggesting that they are independent proteins.

There is a remote possibility that the demeth-
30 ylation observed is not a bona fide demethylation but a consequence of a glycosylase removal of mC, followed by removal of the remaining deoxyribose-phosphate by AP (apyrimidine) nuclease, repair of the gap catalyzed by DNA polymerase using trace dCTP contained in the frac-
35 tion and ligation of the break with ligase in the pres-

ence of residual ATP. For this hypothesis to be con-
sistent with our data, four independent enzymes and two
cofactors have to cofractionate with DNA dMTase. To
exclude the possibility that a trace amount of dCTP is

5   bound to DNA dMTase active fraction, we have added 20
μM of ^{32}P labeled dCTP (10×10^6 cpm) to the nuclear
extract and determined its elution profile on the DEAE
column. Less than background cpm (10 cpm) were
detected in the DNA dMTase active fraction suggesting

10 that our first column purifies dCTP away from the DNA
dMTase at least 1×10^6 fold (Fig. 1B). If any dCTP is
present in the nuclear extract, the remaining concen-
tration after fractionation on DEAE is well below the
Kms of the known DNA polymerases. The possibility that

15 dCTP is so tightly bound to the enzyme that it could
not be replaced by the exogenous ^{32}P labeled dCTP is
very remote since an enzyme using dCTP as substrate
must readily exchange dCTP.

The active fraction 10 was further fractionated

20 sequentially on the following columns: S-Sepharose and
Q-Sepharose. The DNA dMTase eluted at the high salt
fraction from both columns as determined by the
[mdC^{32}pdG]n demethylation assay (Fig. 1A). The ion
exchange chromatography was followed by chromatography

25 on DEAE-Sephacel.

The fact that we have maintained our activity
even after 4 fractionation steps (Table 1) and that
only a single polypeptide is apparent after the last
purification step argues strongly against the possibil-

30 ity that the activity detected in our study is a repair
or replacement activity. Any replacement mechanism
must involve a number of proteins and additional cofac-
tors and substrates. In summary, the chromatography of
the demethylase activity in A459 cells provides strong

support to the hypothesis that mammalian cells bear a *bona fide* demethylase activity.

DNA dMTase releases a volatile derivative

5   A *bona fide* demethylation has to result in release of the methyl group as a volatile derivative such as CO_2, methanol, methane or formaldehyde. We have therefore incubated a {[^{3}H] -CH_3-dCpdG}n double stranded oligonucleotide with the different column fractions and the rate of release of the tritiated

10   methyl from the aqueous phase was determined by scintillation counting of the remaining radioactivity in the reaction mix. As demonstrated in Fig. 1b (diamond), the dMTase active fractions release labeled methyl groups from the methylated substratè.

15

DNA dMTase is a protein which is inhibited by RNA, does not involve an exchange activity and does not require additional cofactors

DNA dMTase activity measured either as transfor-

20   mation of mdC to C (Fig. 2a) or as release of volatile methyl residues (Fig. 2c) is abolished after proteinase K treatment and is not inhibited but rather enhanced following RNase treatment. 500 μM of ddCTP which inhibits DNA polymerase does not inhibit demeth-

25   ylation of the [mdC32pdG]n substrate, nor is it inhibited by high concentrations of methyl-dCTP (500 μM) (Fig. 2a), which is consistent with the hypothesis that demethylation does not involve an excision and replacement mechanism. If a replacement mechanism is involved

30   in demethylation, the presence of mdCTP should result in incorporation of methylated cytosines and essential inhibition of demethylation. Thus, the DNA dMTase identified here is a protein and not an RNA and is unequivocally different from the previously published RNA

35   based or glycosylase based demethylase activities.

The DNA dMTase reaction proceeds without any requirement for additional substrates such as dCTP, redox factors such as NADH and NADPH or energy sources such as ATP (data not shown). As observed in Fig. 2b

5 and 2d, the DNA dMTase reaction maintains its initial velocity up to 90 minutes and continues up to 120 minutes. This time course is inconsistent with dependence on enzyme-bound additional nonreplenishable substrates such as dCTP or ATP or a nonreplenishable redox factor

10 such as NADH or NADPH. Exhausting the nonreplenishable substrate or redox factor would have resulted in rapid deceleration of the initial velocity.

A product of the demethylation reaction is deoxyCyto-
15 **sine in DNA**

What is the product of the demethylation reaction? The results presented above (Fig.1a, 2a and b) based on a one dimension TLC separation show that DNA dMTase generates dC from mdC in DNA. To further sub-

20 stantiate this conclusion, we subjected DNA dMTase treated DNA to remethylation with the CpG MeTase M.*Sss* I which can transfer a methyl group exclusively to dC. The results presented in Fig. 3a show that the demethylated product of DNA dMTase is dC since it is com-

25 pletely remethylated with M.*Sss* I. The identity of the demethylated product as dC was further established by a two-dimension TLC analysis demonstrating that the product of dMTase comigrates with a cold dCMP standard in both dimensions (Fig. 3b).

30 DNA dMTase does not release a nucleotide, a phosphorylated base or phosphate from methylated DNA when incubated with a [32pmdCpdG]n substrate which included a labeled 32P 5' to mdC or our standard methylated substrate (Fig.1) where 32P is 3' to the m5dC

35 (Fig. 3c). Nuclear extracts which obviously contain a number of glycosylases and nucleases release phospho-

rylated derivatives in the same assay (Fig. 3c).
dMTase transforms the methyl cytosine in the
[32pmdCpdG]n substrate to cytosine as demonstrated
when the reacted DNA is digested to 5' mononucleotides
5   (Fig. 3c +V PDS) and analyzed by TLC. Since this
reaction does not involve release of a 32P derivative
(Fig. 3c -V PDS), it demonstrates that dMTase trans-
forms methylated cytosines to cytosines on DNA without
disrupting the integrity of the DNA substrate by glyco-
10   sylase or nuclease activity .

The second product of the dMTase reaction is methanol

What is the identity of the leaving group? The
results presented in Fig1b suggest that the labeled
15   methyl leaves the DNA as a volatile compound. The
demethylase reaction involves release of the methyl
group *per se* whereas the cytosine base ring remains in
the aqueous phase. Fig. 4a demonstrates this point by
using a methylated plasmid labeled with a ^{3}H-hydrogen
20   at the sixth position of cytosine and [14C]-methyl at
the fifth position of cytosine as a substrate.

The three most obvious candidates the methyl
group is leaving as are formaldehyde, carbon dioxide,
and methanol. Methadone trapping for labeled formalde-
25   hyde detection and sodium hydroxide trapping for
labeled carbon dioxide detection were both negative in
identifying the form in which the methyl group is leav-
ing in the dMTase reaction (data not shown). The other
possible chemical form that the methyl group may leave
30   the DNA as, is methanol. Since methanol is a volatile
compound, a simple method to measure generation of
methanol is a scintillation-volatilization assay (see
Fig. 4b for description). Volatilization assays have
been previously used to measure release of methanol in
35   demethylation reactions. The demethylation reaction
mix containing the labeled {[^{3}H] -CH$_3$-dCpdG}n substrate

with either dMTase or no enzyme, as a control, is added
to an uncapped 0.5 ml tube which is placed in a sealed
scintillation vial containing scintillation fluid.
Released methanol is volatile, diffuses out of the open

5   reaction tube and is mixed with the excess of the scin-
tillation fluid in the vial registering as counts in
the scintillation counter. As a control indicating
that methanol is volatilized under the conditions of
our assay, we incubated approximately equal counts of

10   radioactively labeled methanol under the same condi-
tions and measured the counts in a scintillation coun-
ter at different time points. As observed in Fig. 4c
the majority of methanol in the reaction tube volatil-
izes from the reaction tube into the scintillation

15   fluid following an overnight incubation at 37°C. The
experiment shown in Fig. 4b demonstrates that volatil-
ized label is released from methylated DNA only in the
presence of dMTase.

      The identity of the volatile group has been

20   determined to be methanol by a gas chromatography (GC)
analysis. The demethylation and control reactions
(indicated in Fig. 4e) were performed in an uncapped
tube placed in a sealed scintillation vial containing a
larger volume (300μl) of water. The volatile residue

25   diffuses into the surrounding water and mixes with it.
A 2 μl sample of the surrounding water was injected
into a GC column as described in the methods. As
shown in Fig. 4e, the volatile compound released by
dMTase in a dose response manner coelutes with metha-

30   nol. Release of methanol is observed only in the pres-
ence of both dMTase and methylated DNA. No methanol is
released when dMTase is reacted with nonmethylated DNA,
demonstrating that methanol is a product of demethyla-
tion of DNA.

The leaving group was also identified as methanol using gas chromatography coupled with Mass spectrometry (GC-MS). As illustrated in Fig. 4f., incubation of methylated DNA with dMTase (dMTase+ME-DNA)

5   results in release of a peak with the retention time and mass spectrum (peaks are identified at 32 and 29 atomic mass which are the atomic masses of methanol and ionized methanol respectively) which is consistent with its identification as methanol. Incubation of dMTase

10   with nonmethylated DNA does not release methanol indicating that methanol is a product of the demethylation reaction. No methanol is released when the samples are incubated with dMTase treated with protease K indicating that the release of methanol from methylated DNA is

15   catalyzed by an enzymatic activity.

Demethylation involves transfer of a hydrogen from water to regenerate cytosine

If demethylation involves removal of the methyl

20   moiety from mdC, a hydrogen has to be transferred to the carbon at the 5' position to regenerate cytosine. Since no redox factors are involved, what is the source of the hydrogen? To test the hypothesis that the source of the hydrogen is water, we incubated either

25   non labeled [mdCpdG]n or [dCpdG]n double stranded DNA with DNA dMTase for different time periods in the presence of tritiated water, following which the DNAs were digested to 3' dNMPs, separated on TLC with non-radioactive standards for each of the 5 possible dNMPs

30   and exposed to a tritium sensitive phosphorimaging plate. As seen in Fig.4d, dMTase catalyzes the transfer of a tritiated hydrogen from water to dCMP in methylated DNA in a time dependent manner only when methylated DNA is used as a substrate. Based on the

35   experiments described in Fig.3 and 4 we propose that dMTase catalyzes the exchange of the methyl group at

the 5' position of cytosine in DNA with hydrogen from water and the methyl group reacts with the remaining hydroxyl group to form methanol (Fig. 5).

5   Substrate and sequence specificity of DNA dMTase

Methylation of CpG dinucleotides is the most characterized modification occurring in genomic DNA8,48. The results presented in Fig.6 demonstrate that DNA dMTase is a general DNA dMTase activity that

10   demethylates fully or hemimethylated dCpdG in DNA flanked by a variety of sequences which are distributed at different frequencies, but does not demethylate methylated adenines or methylated cytosines that do not reside in the dinucleotide CG. First, as shown in

15   Fig.6a, a plasmid DNA methylated *in vitro* at all dCpdG sites with M.*Sss* I and all d*CdCdGdG sites with M. *Msp* I (which methylates the external C in the sequence *CCGG, thus enabling the determination of demethylation at the CC dinucleotide) and in *vivo* with the *E. coli*

20   DCM MeTase at dCmdCdA/dTdGdG sites and with the DAM MeTase at dGmdAdTdC sites (adenine methylated) was treated with dMTase and the state of methylation of the plasmid was determined using the indicated methylation sensitive restriction enzymes. dMTase demethylates C*G

25   methylated sites as indicated by the sensitivity of the dMTase treated plasmid to *Hpa* II and *Hha* I but does not demethylate C*C,C*A or C*T methylated sites as indicated by the resistance to *Msp* I and *Eco* RII restriction enzymes, or adenine methylation as indicated by

30   its sensitivity to *Dpn* I. Second, bisulfite mapping analysis of methylation of 5 methylated C*G sites residing in a M.*Sss* I *in vitro* methylated pMetCAT plasmid following dMTase treatment shows that all C*G sites are demethylated irrespective of their flanking

35   sequences thus excluding the possibility that demethylation is limited to CCGG or CGCG sequences (Fig. 6b).

Third, dMTase does not demethylate two fully methylated cytosine bearing oligomers [dmC32pdA]n, [mdC32pdT]n demonstrating that mdCpdA and mdCpdT are not demethylated by DNA dMTase (Fig. 6d). Fourth, dMTase demethylates a hemimethylated synthetic substrate [dCpdG]n*[mdC32pdG]n (Fig. 6d). Demethylation of SK is complete under these conditions (Fig. 6a) whereas demethylation of a methylated [mdCpdG]n substrate is not complete under the same conditions (Fig. 6d). This can reflect differences in the sequence composition of the substrate and the frequency of methylated cytosines. The [mdCpdG]n contains on average 16 fold more methylated cytosines per molecule than plasmid DNA. Alternatively, these differences might reflect discrepancies in the assays used, restriction enzyme digestion versus a nearest neighbor analysis. To address this discrepancy we have labeled a fully methylated SK plasmid with $[\alpha^{32}P]$dCTP, 5-methyl-dCTP and the other dNTPs, subjected it to dMTase treatment and digested it to mononucleotides at different time points following the initiation of the reaction and subjected the samples to a TLC analysis. As shown in Fig. 6c, the SK plasmid is fully demethylated at 3 hours which is consistent with the results obtained with methylation sensitive restriction enzymes (Fig. 6a).

The Km of DNA dMTase for hemimethylated and fully methylated DNA was determined by measuring the initial velocity of the reaction at different concentrations of substrate (Table 2). The calculated Km for hemimethylated DNA is 6 nM which is two fold higher than the Km for DNA methylated on both strands, 2.5-3 nM (Table 2). It is unclear yet whether this small difference in affinity to the substrate has any significance in a cellular context. Thus similar to the DNA MeTase DNA dMTase shows dinucleotide sequence

selectivity but in difference from DNA MeTase which shows preference to hemimethylated substrates dMTase prefers fully methylated DNA which is consistent with a role for DNA dMTase in altering established methylation patterns.

Table 1

Purification of DNA dMTase

Purification step	Total protein (µg)	Total dpm	pMole/µg	pMole/µg/h	Fold Purification
Nuclear extract	6000	1107.2	5.5×10^{-5}	1.833×10^{-5}	-
DEAE-Sephadex	3.75	5844	0.4674	0.156	8445.5
SP-Sepharose	0.77	5106	1.989	0.663	35939.84
Q-Sepharose	0.46	5335	3.4	1.13	62860.65
DEAE-Sephacel	0.018	1834	30.57	10.19	552243.2

Table 2

Kinetic parameters for DNA dMTase

Method	K_m (DNA)	V_{max} (pMole/h)
Methylated oligo CpG	2.5 nM	340
Hemi-methylated CpG	6.0 nM	402
Methylated SK-DNA	3.3 nM	40.42

Cloning and construction of demethylase expression vectors

PCR amplification of the MBD domain of the putative demethylase candidate cDNA

One µg of total RNA prepared from the human small lung carcinoma cell line A549 was reverse transcribed using Superscript reverse transcriptase and random primers (Boehringer) in a 25 µl reaction volume according to conditions recommended by the manufacturer (GIBCO-BRL). Five µl of reverse transcribed cDNA were subjected to an amplification reaction with Taq polymerase (Promega, 1 unit) using the following set of

primers: sense   5'CTGGCAAGAGCGATGTC 3'   SEQ   ID   NO:9,
antisense 5'AGTCTGGTTTACCCTTATTTG 3' SEQ ID NO:10.

Amplification conditions were: step 1. 95°C 1
min.; step 2: 94°C 0.5 min; step 3: 45°C 0.5 min.; step
5   4: 72°C 1.5 min; steps 2-4 were repeated 30 times.
$MgCl_2$ was adjusted to 1 mM according to conditions rec-
ommended by the manufacturer.   The PCR products were
cloned in pCR2.1 vector (InVitrogen) and the sequence
of the cDNAs was verified by dideoxy-chain termination
10   method using a T7 DNA sequencing kit (Pharmacia). The
amplified fragment was excised from the plasmid with
EcoRI, labeled with a Boehringer random prime labeling
kit according to manufacturer's protocol and alpha ^{32}P-
dCTP. The labeled probe was used to screen a HeLa cell
15   cDNA library in λTriplEx phage (Clontech) according to
standard procedures.   Positive clones were identified
and further purified by serial dilutions for 4 rounds.
The insert in the pTriplEx plasmid was excised from the
phage according to manufacturer's protocols and the
20   identity of the insert was verified by sequencing. The
insert was excised by NotI restriction and subcloned
into either the inducible expression vector: Retro tet
on (Clontech) in the sense and antisense orientation or
the pcDNA3.1/His Xpress vector in all three frames and
25   in the antisense orientation.

**Transfection and expression of demethylase in verte-
brate cells**

Ten μg of either Retro tet on demethylase or
30   pcDNA 3.1/His Xpress demethylase are mixed with 8 μl of
transfection lypophilic reagent Pfx-2 (Invitrogen) and
placed upon 100,000 mouse (3T3 Balb/c, human (A549) or
monkey cells (CV-1) according to manufacturer's proto-
col in OPTIMEM medium for 4 hours. Cells are harvested
35   after 48 hours and demethylation and demethylase activ-
ity is determined by measuring total genomic DNA meth-

ylation using standard techniques or a cotransfected *in vitro* methylated plasmid using a HpaII /MspI restriction enzyme analysis. Cellular transformation is measured by a soft agar assay.

5

Demethylation of pBluescript SK(+) Plasmid

About 4 μg plasmid pBluescript SK (Stratagene) was subjected to methylation using SssI methylase. The methylated plasmid (4 ng) was incubated for different

10 time points as indicated with 30 μl of DNA dMTase Fraction 4 of DEAE-Sephacel™ column under standard conditions, extracted with phenol: chloroform and precipitated with ethanol. About 1 ng of the plasmid were subjected to digestion with 10 units each of either of

15 the restriction endonuclease EcoRII (GIBCO-BRL), DpnI, or HpaII (New England Biolabs) before and after methylation as well as after DNA dMTase treatment in a reaction volume of 10 μl for 2 hour at 37°C. Following restriction digestion the plasmids were extracted with

20 phenol:chloroform, ethanol precipitated and resuspended in 10 μl. The plasmids were electrophoresed on a 0.8% (w/w) Agarose gel, transferred onto a Hybond™ Nylon membrane and hybridized with pBluescript SK(+) plasmid which was ^{32}P labeled by random-priming (Boehringer

25 Mannheim).

dMTase activity coelutes with a ~45 KDa polypeptide when sized under denaturing conditions but migrates as a higher molecular weight complex under non denaturing

30 **conditions.** dMTase was purified up to 500,000 fold by four chromatographic steps (Table 1). We first determined the identity of the polypeptide associated with dMTase activity by SDS-PAGE analysis of the active fractions. As observed in Fig. 7a, a cluster of 4

35 polypeptide bands from ~44 KDa to 35 KDa coelute with dMTase activity in the last two chromatographic steps

(the lower fragment might be a degradation product as evidenced by its abundance in the later chromatographic steps). However when the active DEAE-Sephacel fraction is size fractionated on a 4% non denaturing acrylamide

5 column, the dMTase activity elutes at the high molecular weight of ~170 KDa (Fig. 7c, fraction 63). SDS-PAGE analysis of this fraction (63) reveals only two bands (Fig. 7b) observed in the active chromatographic fractions (Fig. 7a)   To further determine whether

10 dMTase is found in a multimeric complex, fraction 63 was size fractionated on a glycerol gradient (Fig. 7d) and DNA dMTase activity eluted at the ~170 kDa range. As only two main small polypeptides were identified in fraction 63 (approximately 35-43 KDa), dMTase is proba-

15 bly found in either a homomeric complex if only one of the two peptides is dMTase or a heteromeric complex if both polypeptides are associated with dMTase activity.

a.   Identification of a lead DNA dMTase candidate by
20 **homology search of dbEST**

As the purification of dMTase suggests that the dMTase is of very low abundance, only ~19 ng of dMTase could be isolated from 6 mg of nuclear extract (Table 1), we opted for cloning the dMTase based on its

25 following functional properties. First, since dMTase specifically demethylates methylated CG dinucleotides, we assumed that it should bear the ability to recognize methylated CG dinucleotides. Second, the demethylase transforms methylated cytosine in DNA to cytosine.

30 Third, the demethylase releases the methyl group as a volatile compound.

Previous reports have shown that proteins interacting with methylated DNA share a common domain (MDBD). A TBLASTN search of the dbEST database identi-

35 fied a novel expression tag cDNA (from a T-cell lymphoma Homo sapiens cDNA 5' end) (gb/AA361957/AA361957

EST71295) and the mouse homologue ((gb/W97165/W97165 mf90g05.r1) from Soares mouse embryo NbME13.5) with unknown function that bears homology to the MDBD (Fig. 8a). A search of the GenBank database verified

5 that it is a novel cDNA that has not been included in GenBank. Alignment of the novel EST and MeCP2 and MeCP1 associated protein has revealed no homology beyond the previously characterized MDBD which is consistent with a different function for this methylated

10 DNA binding protein. A 201bp fragment bearing the sequence identified in the search was reverse transcribed and amplified from human lung cancer cell line A549 RNA and was used to screen a cDNA library from Hela cells. The largest insert cloned was of 1.36 kb

15 size and its sequence identity with the EST sequence was determined. The cDNA is novel and has no homologue in GenBank and no function has ever been assigned to it. A virtual translation of the protein identified an open reading frame (ORF) of 262 amino acids (Fig. 8b).

20 The ORF may extend further 5' as no in frame stop codon was found upstream of this ATG. However, RACE analyses and further searches of the dbEST have failed to identify 5' sequences upstream to the one identified in our screening.

25 A BLAST search of the candidate protein using the Predict protein server against a database of protein domain families has identified only the MDBD domain and found no homologue to the sequence in the data base search. No other functional motifs were

30 identified by the Prosite analysis. This is consistent with a novel biochemical function for this protein. A coiled coil prediction of the sequence identified a coiled coil domain which is known to play a role in protein protein interactions.

The identified cDNA encodes an mRNA that is widely expressed in human cells as revealed by a Northern blot analysis of human poly A+ mRNA (Fig. 8c) as one major transcript of ~ 1.6 kb which is close to the size of the cloned cDNA, verifying that the cloned cDNA does not represent a highly repetitive RNA but rather a mRNA encoded by a single or low copy number gene.

In vitro translated candidate cDNA bears dMTase activity

A conclusive proof for the existence of a single protein that *bona fide* demethylates DNA is to demonstrate that an *in vitro* translated candidate cDNA can volatilize methyl groups from methylated DNA and transform a methyl cytosine to cytosine in an isolated system. The candidate dMTase cDNA was subcloned it into a pcDNA3.1/His Xpress (INVITROGEN) expression vector in the putative translation frame (pcDNA3.1His A) and in a single base frame shift (pcDNA3.1His B), and was *in vitro* transcribed and translated in the presence of ^{35}S-methionine and the resulting translation products were resolved by SDS-PAGE. Autoradiography revealed a ~40KDa protein (Fig. 10a). The apparent size of the *in vitro* translated protein is shorter by ~3-5 KDa from the apparent size of the purified protein. The cloned cDNA might be missing some upstream amino acids as discussed above or might be differently modified in human cells.

Two tests established whether the *in vitro* translated candidate cDNA is a *bona fide* dMTase. We first tested whether *in vitro* translated protein (purified on a Ni2+ charged agarose resin) can volatilize and release methyl residues in $[^{3}H]-CH_{3}$-DNA using a radioactive trapping volatilization assay. To verify that the volatilized counts are true ^{3}H counts, a spectrum analysis was performed. As demonstrated in Fig.

10b no volatilization of tritiated methyl residues is observed in the misframe dMTase (misframe) whereas *in vitro* translated putative dMTase cDNA catalyzes the volatilization of ^{3}H-CH$_3$ residues which are trapped in
5   the scintillation cocktail.

Second, *in vitro* translated dMTase cDNA transforms CH$_3$-cytosine residing in [^{32}P]-α-dGTP labeled plasmid DNA or in [methyl-dC32pdG]n double stranded oligomer DNA to cytosine, whereas a frame shift *in*
10 *vitro* translated dMTase does not demethylate DNA (Fig. 10d). This demonstrates that the dMTase activity is dependent on the dMTase translation product and not a contaminating activity found in the *in vitro* translation kit that copurifies with the putative dMTase. The
15 reaction carried out by the *in vitro* translated dMTase displays: dependence on the dose of *in vitro* translated product (Fig. 10c), time dependence (Fig. 10d) and dependence on translated protein (Fig. 10b & d misframe, Fig. 10c protease K treatment). Taken together,
20 these results strongly suggest that the cDNA cloned here codes for a *bona fide* enzymatic DNA demethylase activity.

Transiently transfected dMTase cDNA demethylates DNA
25   dMTase cDNA and the pcDNA3.1HisC vector control were transiently transfected into human embryonal kidney cells to test whether the cDNA can direct expression of dMTase activity in human cells. The His-tagged proteins were bound to Ni2+ agarose resin and eluted
30 from the resin with increasing concentrations of imidazole. The expression of the transfected dMTase was verified by a Western blot analysis (Fig. 11b). The imidazole fractions were assayed for their ability to volatilize and release methyl residues in [^{3}H]-CH$_3$-DNA
35 using a radioactive trapping volatilization assay 1. As observed in Fig. 11a, imidazole fractions from

dMTase transfected cells volatilize[^{3}H]-CH$_3$ whereas no tritiated counts are detected in DNA treated with imidazole fractions from cells transfected with a misframe mutation of dMTase or non transfected cells. The tran-

5   siently expressed dMTase transforms methylated cytosine in DNA to cytosine residing in two different substrates (Figs. 11c & 11d), in a protein dependent manner (Figs. 11c & 11e), and the reaction displays substrate dependence and saturability (Fig. 11f). Transiently

10   expressed dMTase was loaded on a non denaturing glycerol gradient to determine its native MW. Similar to dMTase purified from human cells, cloned and purified dMTase activity fractionated at the 160-190 KDa range (data not shown). This is consistent with self asso-

15   ciation of cloned dMTase possibly mediated by the coiled-coil domain.

Cloned DNA dMTase catalyzes a hydrolysis of 5-methyl-cytosine to release methanol

20   We determined the mechanism by which methyl residues are released by the cloned dMTase (from Fig. 11) and compared it to the purified *bona fide* dMTase activity. Increasing amounts of non labeled [methyl-dCpdG] DNA were incubated with either the *bona fide*

25   dMTase activity purified from A549 cells or the cloned dMTase in the presence of [^{3}H] water for 3 hours followed by digestion to mononucleotides, a thin layer chromatography and autoradiography. As Fig. 12a shows, both reactions replace the methyl group in 5-methylcy-

30   tosine with a proton donated from water as indicated by the presence of [^{3}H] label in cytosine.

The identity of the leaving methyl group in the demethylation reaction catalyzed by the purified bona fide dMTase activity was shown to be methanol. In

35   order to identify the form that the methyl residue leaves as in the demethylation reaction catalyzed by

the cloned dMTase an identical gas chromatography/mass spectrometry analysis of the reaction products was performed as in1. Only the properly translated form of dMTase (both in vitro translated and transiently trans-
5 fected and purified) is able to produce ions character-istic of methanol in a mass spectrometric analysis (mass of 32 and 29, Fig. 12b). These results suggest that the demethylation reaction catalyzed by the cloned dMTase is hydrolysis of the 5-methyl-cytosine to cyto-
10 sine and methanol as described for the purified dMTase1.

DNA dMTase activity is undetectable in nontransformed cells

The assays for dMTase activity described here
15 and the cloning of DNA dMTase cDNA enables a study of its expression at different cellular states. Global hypomethylation of DNA is a common observation in can-cer cells. This has been a perplexing observation, since DNA MeTase activity is elevated in cancer cells.
20 Hyperactivation of DNA MeTase has been proposed to play a role in cancer development. This paradox raises questions on the proposed role of the elevated levels of DNA MeTase in cancer cells. One simple explanation that has been previously suggested to resolve this
25 paradox is that cancer cells express induced levels of DNA dMTase. We compared the DNA dMTase activity in equal concentrations of DEAE-Sephadex fractionated nuclear extracts (fractions 9-10) prepared from a num-ber of carcinoma cell lines H446, Colo 205, Hela, and
30 A549 with a similar preparation from human skin fibro-blast cells at initial rate conditions using [mdC32pdG]n double stranded oligomer as a substrate. As observed in Fig. 13a, whereas DNA dMTase activity is readily observed in all carcinoma cell lines, it is
35 undetectable in nontransformed human cells. The absence of dMTase activity in human primary cells

reflects the situation *in vivo* since dMTase activity is undetectable in preparations from different murine tissues whereas dMTase activity is present in a murine carcinoma cell line P19 that was transfected with the

5 H-Ras protooncogene, or human tumors carried as xenografts in the same strain of mouse (Fig. 1a: COLO 205, A549. Hela). These conclusions were verified using the radioactive-trapping volatilization assay shown in Fig. 13c.

10 Since dMTase mRNA has been detected using a sensitive poly A+ Northern blot in all normal human tissues, we tested the hypothesis that the absence of detected dMTase activity in normal tissues reflects a quantitative difference in DNA dMTase mRNA between nor-

15 mal tissues and cancer lines. A Northern blot analysis and quantification of dMTase mRNA by a slot blot analysis shown in Fig. 13d using total RNA supports this hypothesis. Whereas minute levels of dMTase mRNA are detected in normal tissues, high levels of dMTase are

20 expressed in a murine carcinoma cell line Y1 that bears a 30 fold amplification of *Ha-ras*.

A second DNA demethylase dMTase2 identified in human and mouse

cDNA sequences, predicted amino acid sequences, and

25 GenBank accession numbers of both dMTase1 and dMTase2 from human and mouse are shown. We claim that the high level of identity of the two proteins (Figs 9c and e) suggests that the two proteins can perform the same function, DNA demethylation. The N-terminals of

30 dMTase1 and dMTase2 contain a Methylated DNA Binding Domain (MBD) and near their C-terminals is a coiled-coil domain, however the middle portions of the protein sequences have no homology to any know structural or catalytic motif. Importantly, their middle regions are

35 still extensively homologous suggesting that the cata-

lytic site of the demethylase activity lies in this
area on both proteins.

**Induced expression of DNA demethylase in the Antisense
orientation inhibits tumorigenesis *ex vivo***

5   To test the hypothesis that inhibition of DNA
dMTase can inhibit tumorigenesis tetracycline inducible
vectors carrying the human dMTase1 cDNA in either the
sense or antisense orientation were constructed and
transiently transfected into HEK 293 cells, treated for
10   48 hours either in the presence or absence of doxycy-
cline (a tetracycline analogue), selected for the last
24 hours with puromycin, and then plated on soft agar
and allowed to grow for seven days. After seven days
colonies were scored and the data presented clearly
15   show that doxycycline induced expression of the dMTase1
cDNA in the antisense orientation reduced colony forma-
tion (Fig. 15).

**Imidazole is a small molecule inhibitor of DNA
demethylase activity**

20   A template small molecule, imidazole, was tested
for the ability to inhibit DNA dMTase activity. In a
volatilization of radioactive methyl residues assay,
concentrations from 1μM to 10mM of imidazole were incu-
bated in a typical volatilization of radioactive methyl
25   residues as described above. The graph clearly demon-
strates a dose dependent inhibition of DNA dMTase
activity by imidazole, and validates a rationale for
testing imidazole based molecules as inhibitors of DNA
dMTase activity (Fig. 16).

30   **Identification of DNA demethylase cDNAs and protein
sequences**
Fig. 9a illustrates cDNA sequence of human dMTase1 (SEQ
ID NO:1) and its predicted amino acid sequence (SEQ ID
NO:2), including its Genbank location. **Fig. 9b** illus-
35   trates cDNA sequence of human dMTase2 (SEQ ID NO:3) and
its predicted amino acid sequence(SEQ ID NO:4), includ-

ing its GenBank location. **Fig. 9c** illustrates protein sequence alignment of human dMTase1 and human dMTase2. **Fig. 9d** illustrates cDNA sequence of mouse dMTase1 (SEQ ID NO:5) and its predicted amino acid sequence (SEQ ID

5   NO:6), including its GenBank location. **Fig. 9e** illustrates cDNA sequence of mouse dMTase2 (SEQ ID NO:7) and its predicted amino acid sequence (SEQ ID NO:8), including its GenBank location. **Fig. 9f** illustrates protein sequence alignment of mouse dMTase1 and mouse

10   dMTase2.

      While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any varia-

15   tions, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be

20   applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.